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DNA replication and progression through S phase

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Initiation and completion of DNA replication defines the beginning and ending of S phase of the cell cycle. Successful progression through S phase requires that replication be properly regulated and monitored to ensure that the entire genome is duplicated exactly once, without errors, in a timely fashion. Given the immense size and complexity of eukaryotic genomes, this presents a significant challenge for the cell. As a result, DNA replication has evolved into a tightly regulated process involving the coordinated action of numerous factors that function in all phases of the cell cycle. We will review our current understanding of these processes from the formation of prereplicative complexes in preparation for S phase to the series of events that culminate in the loading of DNA polymerases during S phase. We will incorporate structural data from archaeal and bacterial replication proteins and discuss their implications for understanding the mechanism of action of their corresponding eukaryotic homologues. We will also describe the concept of replication licensing which protects against genomic instability by limiting initiation events to once per cell cycle. Lastly, we will review our knowledge of checkpoint pathways that maintain the integrity of stalled forks and relay defects in replication to the rest of the cell cycle.
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Introduction

Microscopically, it is impossible to notice any discernable change as a mammalian cell transitions from G1 phase of the cell cycle to S phase. There is none of the chromosome acrobatics or morphological metamorphoses that make mitosis so visually distinctive. At the molecular level, however, a dramatic transition occurs as the cell begins the enormous task of duplicating each of the 3.2 billion base pairs that make up its DNA. Massive replication factories scattered throughout the genome are activated and begin unwinding the double helix and building new DNA molecules at a blistering rate of 500 nucleotides per minute with an error rate of one nucleotide in a billion. In prokaryotes, replication begins from a single site and continues until it terminates at the end of the genome. If eukaryotes were to use an identical strategy, however, it would take on the order of several days to completely replicate its significantly larger genome. Therefore, eukaryotic cells initiate replication from multiple locations referred to as replication origins throughout each chromosome. Research in the past two decades has identified an ensemble of more than 20 proteins involved in the process of replication initiation, illustrating the complexity involved in coordinating initiation from hundreds or thousands of origins (Bell and Dutta, 2002; Mendez and Stillman, 2003). The replication process begins with the ordered assembly of a multiprotein complex called the prereplicative complex (pre-RC). Pre-RCs are assembled on DNA at origins in a highly ordered and regulated fashion prior to S phase. During S phase, pre-RCs initiate replication by promoting origin unwinding and facilitating recruitment of the replicative DNA polymerases.

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Events that occur prior to S phase

Assembly of pre-RCs at replication origins

Formation of the pre-RC begins with a group of six related proteins collectively referred to as the origin recognition complex (ORC) that binds to replication origins. Although the proteins that constitute the ORC complex are conserved from yeast to humans, the mechanism by which ORC is directed to origins varies between different eukaryotes (Gilbert, 2001). In the budding yeast *Saccharomyces cerevisiae*, DNA sequences a few hundred base pairs in length, referred to as autonomously replicating sequences (ARS), recruit ORC to DNA (Bell and Stillman, 1992). Within the ARS, ORC recognizes a specific 11 base pair sequence known as the ARS consensus sequence. However, identification of ARS sequences in other eukaryotes has remained elusive, suggesting that origins in other eukaryotes are not defined exclusively by DNA sequence. In the fission yeast *S. pombe*, AT-rich elements rather than specific sequences appear to be sufficient for specifying a functional origin (Okuno *et al*., 1999; Segurado *et al*., 2003). This is consistent

with the finding that *S. pombe* ORC contains multiple AT hook motifs not present in ORC subunits from other eukaryotes (Chuang and Kelly, 1999). In higher eukaryotes, the organization of origins appears even more complex and difficult to define. Human ORC is capable of binding functionally to any DNA sequence (Vashee *et al*., 2003; Schaarschmidt *et al*., 2004), which is consistent with the observation that replication can occur at multiple sites within replication 'zones' that vary in size from 6 to 55 kb in mammalian cells (Dijkwel and Hamlin, 1995). In *Xenopus* egg extracts, initiation is spaced at regular intervals of approximately 10 kb irrespective of sequence (Lucas *et al*., 2000; Blow *et al*., 2001). Recently, it was shown that histone acetylation influences where initiation occurs in *Drosophila* (Aggarwal and Calvi, 2004) and *Xenopus* (Danis *et al*., 2004), indicating that chromatin structure is an important determinant for origin location. Related to these findings, biochemical studies with *Drosophila* ORC reveal a preference for ORC binding to negatively supercoiled DNA over specific sequences (Remus *et al*., 2004), furthering the notion that ORC recognizes three-dimensional structural elements. Defining origins by structure may provide a mechanism for rapidly locating origins in the genome compared to sampling DNA sequence, most of which is nonspecific. Targeting ORC to specific chromosomal locations could also be accomplished though its interaction with other proteins. Cdc6, another pre-RC component, is capable of modulating ORC binding by increasing the stability of ORC on chromatin (Harvey and Newport, 2003) and inhibiting ORC binding to nonspecific DNA (Mizushima *et al*., 2000). Taken together, these findings support a role for epigenetic factors in defining an origin. It has been proposed that this relaxation of sequence requirement for origin specification may be advantageous from an evolutionary perspective as the genome can sustain genetic alterations without concern for losing origins in the process (Gilbert, 2001).

Although the mechanism of ORC recruitment varies between eukaryotes, the subsequent steps involved in assembling the pre-RC after ORC binding are conserved among all eukaryotes. ORC recruits the initiation factors Cdc6 and Cdt1 to origins which are both, in turn, required for loading of the heterohexameric Mcm2–7 complex (Figure 1). Cdc6 belongs to the AAA+ family of ATPases (described in the next section), while Cdt1 bears no sequence homology to any known protein. The crystal structure of a fragment of Cdt1, however, reveals it has a domain that bears structural resemblance to a bacterial replication terminator protein that is believed to physically interact with the bacterial replicative helicase DnaB (Lee *et al*., 2004). Origin unwinding requires the presence of the Mcm2–7 complex (Walter and Newport, 2000) and several lines of evidence indicate that Mcm2–7 functions as the replicative helicase during S phase (Labib and Diffley, 2001; Forsburg, 2004). A subcomplex of Mcm2–7 consisting of Mcm4, 6 and 7 has limited helicase activity *in vitro* (Ishimi, 1997; Lee and Hurwitz, 2001; You *et al*., 2002). However, the six-subunit complex lacks helicase activity *in vitro*, suggesting that Mcm4/6/7 constitutes the core helicase while Mcm2/3/5 are regulatory subunits. This model is supported by studies showing that the ATPase activity of the Mcm2–7 complex requires the coordinated action of all six subunits with Mcm4/6/7 involved in ATP hydrolysis and Mcm2/3/5 regulating its activity (Schwacha and Bell, 2001). A similar mechanism of ATPase organization has been described for the helicase from T7 bacteriophage (Hingorani *et al*., 1997). Complete reconstitution of helicase activity *in vitro* may require proper loading on DNA or activation by other replication factors as is the case *in vivo*. In cells, the Mcm2–7 complex travels with the replication fork during S phase (Aparicio *et al*., 1997) and degradation of Mcm subunits during S phase causes stalling of elongating replication forks (Labib *et al*., 2000), consistent with its role as the replicative helicase. Mcm2–7 complexes are traditionally depicted as being incorporated into the growing pre-RC complex, with two Mcm2–7 complexes loaded at each origin for bidirectional replication. However, recent findings in *Xenopus* favor a model where multiple Mcm complexes are pumped onto the chromatin and spread out from each origin, with each complex having the potential to initiate replication during S phase (Edwards *et al*., 2002). These results are consistent with the finding that the majority of Mcm protein detected by immunofluorescence is associated with unreplicated DNA (Madine *et al*., 1995; Krude *et al*., 1996; Dimitrova *et al*., 1999). Once Mcm complexes are loaded onto chromatin, the other components of the pre-RC are dispensable for replication initiation (Donovan *et al*., 1997; Hua and Newport, 1998; Rowles *et al*., 1999; Maiorano *et al*.,

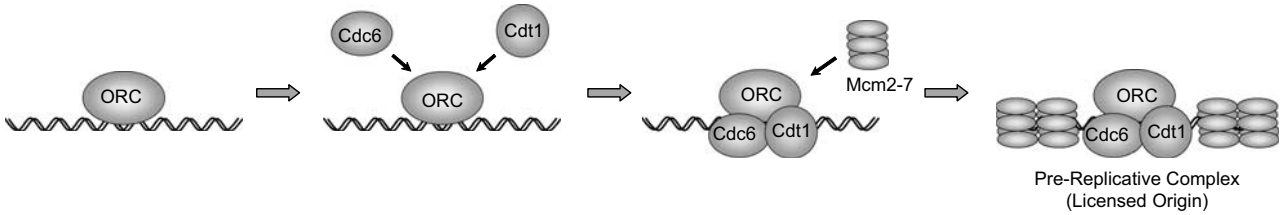

Figure 1 Assembly of the pre-RC. Pre-RC components are recruited to origins in a stepwise manner beginning with the ORC. ORC recruits Cdc6 and Cdt1, which are both required for the subsequent loading of the Mcm2–7 complex. Multiple Mcm2–7 complexes are loaded at each origin and spread out along the chromatin. Pre-RC formation occurs during late M and early G1 phases of the cell cycle and licenses the DNA for replication during S phase

2000; Harvey and Newport, 2003). Therefore, the primary function of ORC, Cdc6 and Cdt1 in replication is to load the Mcm2–7 complex.

Structures of pre-RC homologues from viruses, bacteria and archaea provide our first glimpse into the molecular mechanism of replication initiation in eukaryotes

Although the structures of eukaryotic DNA initiation proteins have yet to be solved, mechanistic insight can be gained from the structures of homologous proteins from other domains of life. The crystal structure of the N-terminus of the Mcm complex from the archaea *Methanobacterium thermoautotrophicum* has recently been determined (Fletcher *et al*., 2003). The archaeal Mcm complex consists of single subunit that forms a head-to-head dimer of homohexamers with a positively charged central pore large enough to encircle single- or double-stranded DNA. Similarly, the eukaryotic Mcm2–7 complex forms a heterohexameric complex with a central pore as determined by electron microscopy (Adachi *et al*., 1997; Prokhorova and Blow, 2000; Sato *et al*., 2000). Although the archaeal crystal structure was lacking the C-terminus containing the conserved helicase and ATPase domains, it is likely that the C-terminal domains from different subunits of the complex oligomerize to function as a hexameric helicase analogous to the viral replicative helicase, SV40 large T antigen (Li *et al*., 2003). The structure of the hexameric SV40 large T antigen is organized into two distinct domains. The N-terminus forms a ring with a central pore large enough to encompass double-stranded DNA, while the C-terminus domain forms a hexameric ATPase (Li *et al*., 2003). Based on the structure, it has been proposed that the two domains twist about each other, causing the central channel to expand and constrict, which distorts the double helix and promotes DNA unwinding. Given the structural similarities to the viral helicase, the archaeal and eukaryotic Mcm complexes probably operate through a similar mechanism. These structures also provide further evidence that Mcm2–7 is the eukaryotic replicative helicase.

The solved crystal structure of an archaeal Cdc6/Orc1 homologue from *Pyrobaculum aerophilum* confirmed that Cdc6 belongs to the same family of AAA+ ATPases as the group of proteins referred to as clamp loaders (Liu J *et al*., 2000). Clamp loaders are found in all domains of life and couple ATP binding and hydrolysis with the opening and closing of ring-shaped molecules, or clamps, around DNA (Davey *et al*., 2002). Since nucleotide binding and hydrolysis is also required for Cdc6-dependent loading of the Mcm2–7 complex (Perkins and Diffley, 1998; Weinreich *et al*., 1999), it has been hypothesized that Cdc6 is the clamp loader for the Mcm2–7 complex. Orc1, 4 and 5 subunits also belong to the AAA+ ATPase family and electron micrographs of the *S. cerevisiae* ORC complex have shown that it undergoes a conformation shift in the presence of single-stranded DNA and ATP (Lee *et al*., 2000), similar to what is seen with the clamp loader RFC (Shiomi *et al*., 2000). Therefore, ORC and Cdc6 may function together as a clamp loader complex for opening and closing Mcm2–7 around DNA at origins.

The archaeal Cdc6/Orc1 structure also revealed the presence of a wing helix domain (WHD) in the C-terminus of Cdc6 (Liu J *et al*., 2000). WHDs belong to the helix turn helix (HTH) family of proteins and have been shown to mediate protein–protein and DNA–protein interactions. Mutations in the WHD domain abrogated Cdc6 function in *S. pombe*, suggesting an essential role in pre-RC formation (Liu J *et al*., 2000). The WHD domain of archaeal Cdc6/Orc1 is essential for binding the archaeal Mcm complex and regulating its helicase activity (Shin *et al*., 2003). Whether the same holds true for eukaryotic Cdc6 has yet to be determined. Surprisingly, when the structure of the bacterial replication initiator protein, DnaA, was solved, it bore a striking resemblance to the archaeal Cdc6/Orc1 structure (Erzberger *et al*., 2002). Despite sharing almost no sequence similarity, both structures consist of a structurally conserved AAA+ domain linked to a HTH domain. In *Escherichia coli*, DnaA uses the HTH domain to bind DNA and position itself at origins with the ATPase domains of neighboring DnaA molecules oriented to form a six-subunit oligomer (Messer *et al*., 2001). Once assembled, the oligomer then functions as a molecular machine to catalyse the melting of DNA and loading of the replicative helicase. Could Cdc6 or ORC function in a similar fashion? Based on the structural conservation between initiator proteins, it is tempting to speculate that this mechanism of origin recognition and processing may be conserved in bacteria, archaea and eukaryotes. Confirmation awaits elucidation of the structures of eukaryotic pre-RC components.

Spatial and temporal regulation of origin firing

Although each assembled pre-RC has the potential to initiate replication, only a subset of these origins is actually triggered to fire during S phase (Santocanale and Diffley, 1996; Walter and Newport, 1997; Okuno *et al*., 2001; Anglana *et al*., 2003). Having more pre-RCs than necessary ensures that the entire genome is replicated even if some pre-RCs fail to initiate. Origins that are normally dormant are activated if neighboring origins fail to fire (Santocanale *et al*., 1999; Vujcic *et al*., 1999). Without these 'backup' origins, failed initiation events create large intervals between origins which increases the distance forks must travel to complete replication. In addition to requiring more time, the probability of fork stalling is increased if forks are required to traverse larger distances. It has been shown in *S. cerevisiae* that removing ARS elements from a chromosome leads to its eventual loss, presumably because the replication forks cannot finish replicating the chromosome during S phase (Dershowitz and Newlon, 1993). Successful initiation also inhibits the firing of nearby origins (Marahrens and Stillman, 1992; Brewer and Fangman, 1994; Anglana *et al*., 2003) by passive replication through surrounding origins (Santocanale *et al*., 1999; Vujcic *et al*., 1999). Therefore origins are activated and suppressed depending on the activity

of neighboring origins, ensuring that an adequate number of origins are activated during S phase.

Initiation is also temporally regulated with each origin firing at a specific time during S phase. Studies have determined that the spatial and temporal programs for origin firing are established at distinct times during G1 phase (Wu and Gilbert, 1996; Raghuraman *et al*., 1997; Okuno *et al*., 2001). The timing decision point (TDP) occurs early during G1 after pre-RC formation. At the TDP, early replicating domains are determined concomitantly with the restructuring of the nuclear architecture after mitosis. Several hours after the TDP, origins that will fire in S phase are selected from among the early and late domains at the origin decision point (ODP). The ODP is also distinct from the Restriction point (R point) during G1 when progression through the cell cycle becomes independent of mitogenic stimuli (Wu and Gilbert, 1997). The physiological significance of the TDP and ODP is poorly understood. The ODP in mammalian cells can be bypassed without affecting progression through S phase (Wu *et al*., 1998). It has been speculated that the TDP and ODP are involved in coordinating chromosomal replication to other cellular processes such as gene expression. In support of this, during development, origin specification begins at the midblastula stage, which coincides with the onset of transcription (Hyrien *et al*., 1995; Sasaki *et al*., 1999). Prior to the midblastula stage, embryonic cells go through rapid rounds of S and M phase with replication occurring at random locations simultaneously suggesting that they lack both a TDP and an ODP. Recently, it has also been suggested that nucleotide availability influences selection of origins at the ODP (Anglana *et al*., 2003).

What is the molecular basis by which origins are selected at the TDP and ODP? For some time, it has been known that early-replicating regions of DNA are associated with euchromatin, while late-replicating regions are localized to heterochromatin. Relocating early origins to heterochromatin is sufficient to convert it to a late firing origin (Ferguson and Fangman, 1992; Friedman *et al*., 1996), and disrupting heterochromatin formation causes late origins to replicate early (Stevenson and Gottschling, 1999). Therefore, replication timing has been attributed to transcription and/or the local chromatin environment. A genomewide study of origin timing in *Drosophila* cells confirmed a significant correlation between time of origin firing and transcription – genes that replicate early are transcribed to higher levels. Although transcription and replication do not occur simultaneously on the same DNA (Wei *et al*., 1998), actively transcribed regions of DNA tend to be hyperacetylated, resulting in an open chromatin conformation which may make the DNA more accessible to replication factors. Consistent with this hypothesis, deletion of the histone deacetylase, Rpd3, in *S. cerevisiae* results in global histone hyperacetylation, with late origins firing earlier in S phase (Vogelauer *et al*., 2002). Furthermore, artificially directing a histone deacetylase to an origin converts an early-firing origin into a late-firing origin (Zappulla *et al*., 2002). Similar experiments were recently carried out in *Drosophila*, although it is not clear if acetylation exerts its effects by recruiting ORC or by activating pre-RCs (Aggarwal and Calvi, 2004). However, it was shown in *Xenopus* eggs that origin firing occurs preferentially at sites of histone acetylation despite indiscriminate ORC binding (Danis *et al*., 2004). Since the TDP occurs concomitantly with the restructuring of the nuclear architecture after mitosis (Li *et al*., 2001), it is also plausible that nuclear positioning also plays a role in replication timing.

Transition into S phase

The transition from G1 to S phase involves the conversion of pre-RCs into replication forks. Initiation requires origin unwinding, stabilization of single-stranded DNA, and loading of the replicative polymerases. These processes require the function of a second set of replication factors and the activities of at least two kinases – cyclin dependent kinases (CDKs) and the Dbf dependent kinase (DDK) (Figure 2). Unlike pre-RCs which form at early and late origins simultaneously, these factors are temporally regulated throughout S phase, being associated with origins at the time of activation. Therefore, mechanisms influencing origin choice and timing probably regulate the targeting of these factors to origins. The majority of work in recent years has focused on identifying the relevant players involved in activating pre-RCs, determining the order in which they associate with origins, and characterizing their cell cycle regulation. The challenge in the future will be to decipher how these factors function together to promote fork assembly and progression during S phase.

Recruitment of initiation factors

The earliest initiation factor recruited to pre-RCs is Mcm10, which is involved in several critical functions. Firstly, along with CDK and DDK activity, Mcm10 loading is required for the recruitment of Cdc45 (Wohlschlegel *et al*., 2002; Gregan *et al*., 2003; Sawyer *et al*., 2004). Cdc45 recruitment is essential for the subsequent origin unwinding (Mimura *et al*., 2000; Walter and Newport, 2000; Masuda *et al*., 2003) and loading of the replicative polymerases (Mimura and Takisawa, 1998; Zou and Stillman, 2000; Uchiyama *et al*., 2001). Secondly, Mcm10 facilitates DDK phosphorylation of Mcm2–7 by physically interacting with both complexes (Lee JK *et al*., 2003). The interaction between Mcm10 and the Mcm2–7 complex has been shown to be important for initiation (Merchant *et al*., 1997; Homesley *et al*., 2000; Izumi *et al*., 2000; Kawasaki *et al*., 2000) suggesting that Mcm10-mediated phosphorylation of Mcm2–7 may play a role in activating the Mcm2–7 complex. Thirdly, mutations in yeast Mcm10 show defects in completion of S phase after origins have fired suggesting a role in elongation (Merchant *et al*., 1997; Kawasaki *et al*., 2000; Gregan *et al*., 2003). Since Cdc45 incorporation into replication

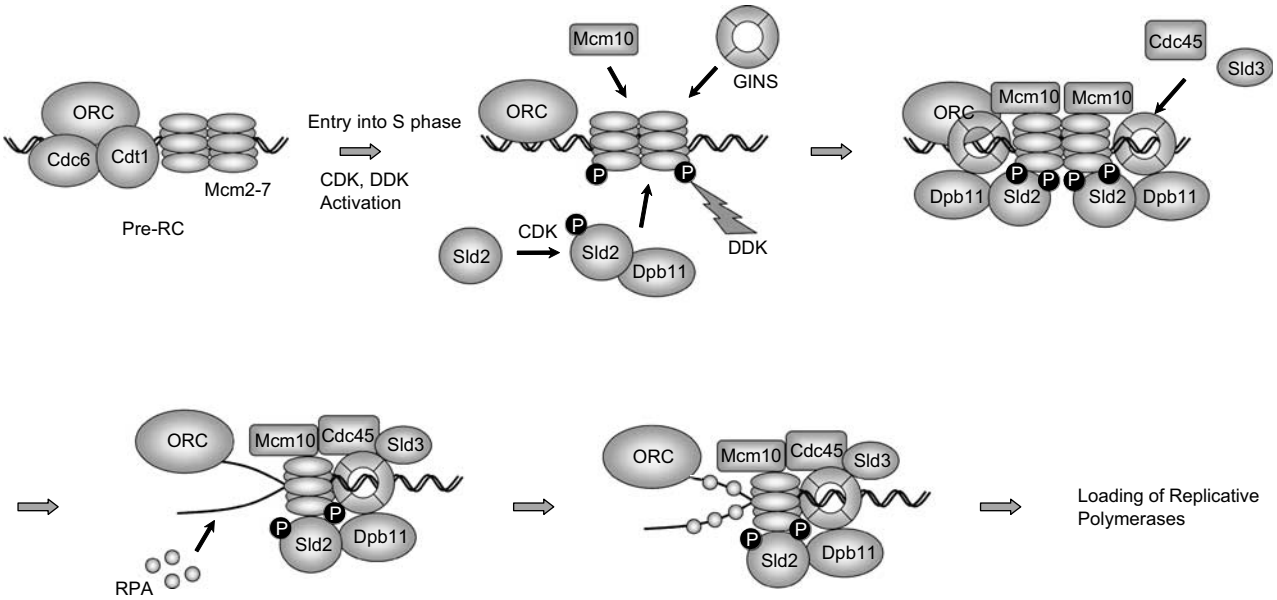

Figure 2 Activation of pre-RCs during S phase involves the assembly of a second set of factors as well as the activity of CDKs and Dbf4 dependent kinases (DDKs). The names of factors used in the figure are from *S. cerevisiae*, but homologous proteins have either been identified in other eukaryotes or are presumed to exist. The order of assembly and interactions between multiple components are not well defined. Appearance of a factor before another factor does not always imply that the first factor is required for the latter. For example, although CDK appears before MCM10 is loaded on the chromatin, the loading event is not dependent on CDK activity. Known targets of CDK and DDK are indicated, but there are probably other targets that have yet to be identified

forks is required for fork progression, Mcm10's function in elongation may be related to its ability to retain Cdc45 in elongating forks. Mcm10 also interacts with the elongation factors, DNA polymerase ε, DNA polymerase δ, DNA2 (Kawasaki *et al*., 2000; Liu Q *et al*., 2000a) and has been shown to activate the primase activity of DNA polymerase α *in vitro* (Fien *et al*., 2004). Mcm10 also plays a role in the ability of active replication forks to pass through uninitiated pre-RCs. Elongating forks pause at unfired pre-RCs in a Mcm10 mutant in *S. cerevisiae* (Homesley *et al*., 2000), suggesting that assembled pre-RCs may present a barrier to fork progression that is overcome though the action of Mcm10.

Although it is clear that Mcm10 is required for initiation, Mcm10 has also been implicated in pre-RC formation in *S. cerevisiae*. Stable association of the Mcm2–7 complex requires the continued presence of Mcm10 on chromatin (Homesley *et al*., 2000). However, this seems unique to *S. cerevisiae*, as experiments in *S. pombe* and *Xenopus* demonstrate pre-RC formation that is independent of Mcm10 (Wohlschlegel *et al*., 2002; Gregan *et al*., 2003). It has been hypothesized that Mcm10 may be required for the maintenance of pre-RCs in *S. cerevisiae* rather than their establishment at origins (Lei and Tye, 2001). Additional differences in the behavior of Mcm10 between eukaryotes include the mechanism of recruitment to chromatin and its regulation during the cell cycle. In *Xenopus*, recruitment requires the prior loading of the Mcm2–7 complex (Wohlschlegel *et al*., 2002), whereas, in yeast, Mcm10 binds chromatin independently of Mcm2–7, but requires the presence of ORC on chromatin (Homesley *et al*., 2000; Gregan *et al*., 2003). Regulation of Mcm10 in mammalian cells is cell cycle dependent, with protein levels and chromatin association highest during S phase (Izumi *et al*., 2000, 2001). However, Mcm10 protein levels in yeast remain constant, with Mcm10 constitutively bound to the chromatin fraction in all phases of the cell cycle (Homesley *et al*., 2000; Kawasaki *et al*., 2000; Gregan *et al*., 2003). Interestingly, the differences in cell cycle regulation between eukaryotes are similar to those seen with ORC. ORC is constitutively bound to origins in yeast, but show a dynamic association with origins during the cell cycle in mammalian cells (DePamphilis, 2003). Therefore, the differences in Mcm10 regulation may be fundamental to the differences in the mechanism of replication between unicellular and multicellular eukaryotes. Alternatively, Mcm10 is tightly associated with chromatin in yeast, but is localized at origins only during S phase. Since Mcm10 is the earliest factor recruited to pre-RCs, regulation of Mcm10 recruitment may be the underlying mechanism that governs replication timing and selection.

As mentioned above, Cdc45 is required for recruitment of the replicative polymerases. Cdc45 interacts with components of the pre-RC and polymerases, providing a physical link between initiation and elongation factors (Hopwood and Dalton, 1996; Dalton and Hopwood, 1997; Zou *et al*., 1997; Kukimoto *et al*., 1999). Cdc45 associates with origins in a CDK- and DDK-dependent manner being loaded at early origins in early S phase, and late origins in late S phase (Aparicio *et al*., 1997, 1999; Mimura and Takisawa, 1998; Zou and Stillman, 1998; Jares and Blow, 2000). In *Xenopus* extracts, an initial origin unwinding event is detected

after Cdc45 binding prior to the association of the single-stranded binding protein RPA and DNA pol α (Walter and Newport, 2000). Formation of a tight complex between Cdc45 and Mcm2–7 closely correlates with helicase activation, suggesting that Cdc45 functions to activate the helicase activity of the Mcm2–7 complex (Masuda *et al*., 2003). Stabilization of single-stranded DNA by RPA further stimulates origin unwinding (Walter and Newport, 2000). After origin firing, Cdc45 leaves the origin and travels with the Mcm complex and components of the replicating fork (Aparicio *et al*., 1997). Degradation of Cdc45 during S phase results in fork stalling, indicating that Cdc45 is also required for the elongation phase of replication (Tercero *et al*., 2000). Stalled replication forks recover if Cdc45 is reintroduced, indicating that Cdc45 can be recruited to forks which have already initiated and left the origin. This is in contrast to Mcm subunits which cause irreversible fork stalling if degraded during elongation (Labib *et al*., 2000). Therefore, Cdc45 may be required for loading polymerases at origins as well as loading or maintaining polymerases at elongating or stalled forks. It will be interesting to determine if polymerases are present at stalled forks in the absence of Cdc45. It has also been proposed that Cdc45 coordinates helicase activity with polymerase activity (Tercero *et al*., 2000). An attractive model would be one where the Mcm helicase is inactive unless bound to the polymerase through Cdc45. Therefore, helicase activity is tightly coupled to polymerase activity, ensuring that the helicase does not begin unwinding DNA without a trailing polymerase. Although speculative, this is highly reminiscent of replication in *E. coli*, where the activity of the prokaryotic helicase is stimulated through a direct interaction with the tau subunit of pol III (Kim *et al*., 1996).

A set of successive genetic screens in *S. cerevisiae* has recently identified an additional set of proteins involved in the transition from pre-RCs to elongating forks. The first screen was designed to find interactors with DNA pol ε, and isolated Dpb11, a subunit of DNA polymerase ε (Araki *et al*., 1995). Dpb11 and its counterpart in *S. pombe*, Cut5, are essential for replication initiation and checkpoint activation (Saka and Yanagida, 1993; Araki *et al*., 1995; McFarlane *et al*., 1997). It was later demonstrated that Dpb11 is in a complex with DNA pol ε and is required for recruiting DNA pol ε and DNA pol α to origins after Cdc45 and RPA binding (Masumoto *et al*., 2000). Putative homologues of Dpb11 have been identified in higher eukaryotes including Mus101 in *Drosophila* (Yamamoto *et al*., 2000), TopBP1 in mammals (Makiniemi *et al*., 2001) and Cut5/Mus101 in *Xenopus* (Van Hatten *et al*., 2002; Hashimoto and Takisawa, 2003), based on their conservation of repeating BRCT protein motifs, and their involvement in both DNA replication and checkpoint activation. The *Xenopus* homologue Cut5/Mus101 has also been shown to be essential for polymerase loading and both the yeast and *Xenopus* proteins are dispensable for elongation (Masumoto *et al*., 2000; Van Hatten *et al*., 2002; Hashimoto and Takisawa, 2003). However, in contrast to yeast, *Xenopus* Cut5/Mus101 binds prior to Cdc45 and is required for Cdc45 binding to chromatin (Van Hatten *et al*., 2002; Hashimoto and Takisawa, 2003). Given the low sequence similarity between the *Xenopus* and yeast proteins, it is possible that *Xenopus* Cut5/Mus101 is not the functional homologue of yeast Dpb11/Cut5. Interestingly, *Xenopus* Cut5/Mus101 recruitment to origins occurs during S phase, but does not require Mcm loading (Van Hatten *et al*., 2002), suggesting that two independent pathways are required for Cdc45 loading – a pre-RC-dependent pathway, and a Cut5/Mus101-dependent pathway.

To find interactors with Dpb11, a second screen was performed which isolated six genes *Sld1–6*, for synthetically lethal with Dpb11 (Kamimura *et al*., 1998). Sld1, 4, and 6 corresponded to known genes previously shown to be involved in replication: Dpb3 (subunit of DNA pol ε), Cdc45 and the checkpoint protein Rad53, respectively. Sld2 was identical to Drc1 (DNA and replication checkpoint 1), which was isolated in an independent screen for Dpb11 interactors in *S. cerevisiae* (Wang and Elledge, 1999) and forms a complex with Dpb11 that is required for replication (Kamimura *et al*., 1998; Wang and Elledge, 1999). Sld3 and 5 represented two novel genes potentially involved in DNA replication. Soon thereafter, Sld3 was shown to be essential for replication in yeast and functions in a complex with Cdc45 (Kamimura *et al*., 2001; Nakajima and Masukata, 2002). Binding of Sld3 to origins is mutually dependent on Cdc45 and both are required for loading of the replicative polymerases (Kamimura *et al*., 2001). After initiation, Sld3 travels with Cdc45 and the elongating fork, further supporting the notion that Sld3 and Cdc45 function together as a complex on chromatin (Kamimura *et al*., 2001). Since Cdc45 is conserved among all eukaryotes, it is expected that Sld3 is present in higher eukaryotes as well, however, functional homologues have yet to be identified. Mutants of Sld5 were used as the starting point for another series of genetic screens, which led to the discovery of the GINS complex consisting of Sld5 and Psf1–3 (Partners of Sld5 1–3) (Takayama *et al*., 2003). The GINS complex forms a ring-like structure in solution (Kubota *et al*., 2003), associates with origins during S phase in a pre-RC- and CDK-dependent manner, and is required for Cdc45 and polymerase loading (Kubota *et al*., 2003; Takayama *et al*., 2003). Chromatin immunoprecipitation of GINS indicates that it associates with DNA neighboring origins after initiation, suggesting a role in replicating forks. However, unlike other factors involved in elongation, GINS mutants still complete S phase after origin firing, albeit more slowly. Experiments using null alleles of GINS subunits are needed to clarify its role in elongation. Given its ring structure and interaction with polymerases, the GINS complex may function similar to PCNA as a processivity clamp for polymerases or to coordinate the action of multiple polymerases.

Activation of CDK and DDK

Initiation requires the activity of two cell cycle-regulated kinases, Cdc7, and cyclin dependent kinase (CDK)

during S phase (Kelly and Brown, 2000; Bell and Dutta, 2002; Masai and Arai, 2002; Kim *et al*., 2003). CDKs intrinsically possess low levels of kinase activity and require activation by a group of proteins known as cyclins, the levels of which oscillate during the cell cycle. In addition to its well-established role in driving the cell cycle from one stage to the next, CDK activity is required for initiation of replication. Many replication factors have been shown to be phosphorylated by CDKs *in vitro* and *in vivo* (for a review, see Kelly and Brown, 2000). However, substrates that are positively regulated by CDK activity have eluded discovery until very recently. Isolated from the screen for Dpb11 interacting proteins described earlier, Sld2 is the first protein identified whose phosphorylation by CDK is required for the initiation of replication in yeast (Masumoto *et al*., 2002; Noguchi *et al*., 2002). Phosphorylation of Sld2 mediates its interaction with Dpb11 and formation of the Sld2–Dpb11 complex is required for its loading at origins and the recruitment of replicative polymerases. No homologues of Sld2 have been found in higher eukaryotes, but given the conservative nature of the proteins that interact with Sld2, it is assumed that a functional homologue exists.

Similar to CDKs, Cdc7 kinase activation requires binding to a noncatalytic subunit known as Dbf4 and the complex is referred to as DDK. DDK activity is also regulated by the periodic stabilization and destruction of Dbf4 protein during the cell cycle, with levels increasing during the transition to S phase (Brown and Kelly, 1999; Cheng *et al*., 1999; Kumagai *et al*., 1999; Oshiro *et al*., 1999; Takeda *et al*., 1999; Weinreich and Stillman, 1999; Ferreira *et al*., 2000). DDK acts at individual origins throughout S phase to activate early- and late-firing origins (Bousset and Diffley, 1998; Donaldson *et al*., 1998). It was demonstrated that *Xenopus* Dbf4 is loaded onto chromatin in a pre-RC-independent manner (Jares *et al*., 2004) and recruits Cdc7 to chromatin. Therefore, Cdc7 is simultaneously activated and localized. However, observations in *S. cerevisiae* and mammalian cells indicate that Cdc7 can associate with chromatin independently of Dbf4 (Weinreich and Stillman, 1999; Sato *et al*., 2003), suggesting that in other eukaryotes Dbf4 activates Cdc7 that is already present on chromatin. Another activating partner of Cdc7 has been cloned in *Xenopus* and humans called Drf1, for Dbf4-related factor (Montagnoli *et al*., 2002; Yanow *et al*., 2003). Although the cell cycle regulation of Drf1 resembles Dbf4 (Montagnoli *et al*., 2002), Drf1 is not required for replication initiation, but instead plays a role in a checkpoint pathway by inhibiting association of Cdc45 with chromatin in response to damage (Yanow *et al*., 2003). Therefore, it has been proposed that Dbf4–Cdc7 and Drf1–Cdc7 phosphorylate different subsets of proteins under different conditions. However, it is still possible that they have overlapping functions, as the effect of depleting both Dbf4 and Drf1 has not been examined.

An abundance of genetic and biochemical data indicates that the Mcm2–7 complex is the physiological substrate for DDK required for replication initiation. Multiple subunits of the Mcm2–7 complex have been shown to be phosphorylated by DDK *in vitro* and *in vivo* (Lei *et al*., 1997; Sato *et al*., 1997; Brown and Kelly, 1998; Kumagai *et al*., 1999; Takeda *et al*., 1999, 2001; Jares and Blow, 2000; Kihara *et al*., 2000). A point mutation in a highly conserved proline in Mcm5 to leucine bypasses the need for DDK activity for replication in *S. cerevisiae* (Hardy *et al*., 1997). Genomic footprinting of this Mcm5 mutant revealed structural changes at origins that resemble those that occur in S phase after Cdc7 activation, suggesting that the Mcm5 mutant was prematurely activated, thereby forgoing the need for DDK (Geraghty *et al*., 2000). When the structure of the archaeal homologue of Mcm2–7 was solved, the conserved proline was found to be buried in a region between two closely packed domains (Fletcher *et al*., 2003). Therefore, replacing the proline with a bulky side chain is predicted to alter the structure of the protein by pushing apart the two domains. Consistent with this hypothesis, mutating the proline of *S. cerevisiae* Mcm5 to residues with small side chains was unable to bypass the requirement for DDK, whereas mutations to large side chains showed effective DDK bypass (Fletcher *et al*., 2003). Since Cdc45 loading requires DDK activity, it is possible that Cdc45 binds to the Mcm2–7 complex after it is phosphorylated and activated by DDK (Jares and Blow, 2000; Walter, 2000; Zou and Stillman, 2000). The order in which CDK and DDK act during the process of replication initiation remains unclear. In yeast, CDK activity is required before DDK (Nougarede *et al*., 2000), while in *Xenopus* DDK activity appears to be required prior to CDK activity (Jares and Blow, 2000; Walter, 2000). It has also been suggested that CDK and DDK are involved in two parallel pathways that are both required for Cdc45 loading (Van Hatten *et al*., 2002). The latter model is attractive because it implies that replication initiation, specifically the loading of Cdc45, requires two independent signals: a replication signal involving pre-RC formation and DDK activity, and a cell cycle signal propagated through CDK activity.

Loading of the replicative polymerases

The final step in replication initiation is the loading of the replicative polymerases (reviewed in Waga and Stillman, 1998; Kawasaki and Sugino, 2001; Hubscher *et al*., 2002). At least three polymerases are essential for replication, DNA pol α, DNA pol δ and DNA pol ε. Although all three polymerases share a conserved catalytic core, they perform specialized functions in elongation. After origin unwinding stimulated by Cdc45 and RPA binding (Mimura *et al*., 2000; Walter and Newport, 2000), DNA pol α is recruited to origins and synthesizes short RNA primers for leading and lagging strand synthesis. DNA pol α is the only polymerase that can initiate synthesis *de novo* on single-stranded DNA, whereas DNA pol δ and DNA pol ε require a primed template for activity. After primer synthesis, polymerase switching occurs, which replaces DNA pol α with DNA pol δ and/or DNA pol ε, which display greater

processivity and have proofreading exonuclease activity. Processive DNA synthesis requires DNA pol δ and DNA pol ε to associate with the ring-shaped processivity factor PCNA (Jonsson and Hubscher, 1997; Mossi and Hubscher, 1998). PCNA encircles DNA and topologically links the polymerase to DNA. PCNA is loaded onto the primed DNA template (Mimura *et al*., 2000) by the clamp loader, RFC, which belongs to the same class of ATPases as Cdc6 and several of the ORC subunits (Ellison and Stillman, 2001). DNA pol δ and DNA pol ε are both essential for proper replication and perform nonredundant essential functions (Waga *et al*., 2001; Fukui *et al*., 2004). Mutants of DNA pol δ and DNA pol ε that are defective in proofreading accumulate mismatches on different DNA strands, suggesting that they are responsible for synthesizing opposite strands of DNA (Morrison and Sugino, 1994; Shcherbakova and Pavlov, 1996; Karthikeyan *et al*., 2000). However, the N-terminus of DNA pol ε, containing the catalytic domain, is not necessary for viability but the C-terminal region involved in protein–protein interaction and checkpoint activation is sufficient (Dua *et al*., 1999; Kesti *et al*., 1999; Feng and D'Urso, 2001). Although this does not rule out a role for DNA pol ε in elongation, these findings suggest that its polymerase activity can be functionally replaced by DNA pol δ. Experiments in *Xenopus* extracts confirm that even in the absence of DNA pol ε, DNA pol δ is capable of performing a significant amount of processive leading and lagging strand synthesis (Fukui *et al*., 2004). In contrast, DNA pol ε fails to significantly elongate primed template in the absence of DNA pol δ (Fukui *et al*., 2004), suggesting that DNA pol δ is the main polymerase for both leading- and lagging-strand synthesis. DNA pol δ has also been shown to perform both leading- and lagging-strand synthesis in the synthesis of the SV40 viral genome (Waga and Stillman, 1998). So the question remains, what is the essential function of DNA pol ε? DNA pol ε loading occurs prior to DNA pol α loading (Masumoto *et al*., 2000), suggesting that it may play a role in origin initiation after pre-RC formation. This would be consistent with the finding that DNA pol ε is not required for SV40 replication, which uses large T-antigen for origin unwinding in place of pre-RCs (Zlotkin *et al*., 1996; Pospiech *et al*., 1999). Since the C-terminus is involved in checkpoint control (Navas *et al*., 1995), DNA pol ε may monitor replication fork progression by augmenting the action of the other polymerases. This is supported by recent genetic studies that have implicated a role for DNA pol ε in correcting errors made by DNA pol α and DNA pol δ (Pavlov *et al*., 2004).

An important component of eukaryotic chromosomes is the histones which contain epigenetic information, or 'epigenome', that must also be passed on to future generations. During replication, histones are divided among the parent and daughter strands and new histones are assembled by chromatin assembly factors (CAFs) that travel with the replication fork (Shibahara and Stillman, 1999; Zhang *et al*., 2000; Hoek and Stillman, 2003). How the histone modifications, which contain information for regulating gene expression, are then copied onto the newly assembled histones on DNA is an intense area of current research (for recent reviews, see McNairn and Gilbert, 2003; Ehrenhofer-Murray, 2004 and references therein).

Replication licensing

Until this point we have discussed the ordered series of events that lead to origin firing during S phase. But how does a cell ensure that each origin fires only once per cell cycle? Repeated rounds of initiation result in re-replicated DNA, leading to chromosomal breakage and genomic instability. Clues to our understanding of how this is accomplished came from early cell fusion experiments (Rao and Johnson, 1970). When a G1 cell is fused to an S phase cell, the G1 nucleus begins to replicate prematurely. Therefore, a G1 nucleus is competent to replicate, but lacks factors present in S phase that are necessary for origin firing. However, delivering these factors to a G2 nucleus by fusing an S and G2 cell fails to initiate replication in the G2 nucleus, indicating that the cell has some means of differentiating between replicated and unreplicated DNA. These observations led to the concept of replication licensing (Blow and Hodgson, 2002; Nishitani and Lygerou, 2002). It was hypothesized that DNA is altered in some fashion during G1 that 'licenses' it for replication. Passage through S phase removes the license, making the DNA refractory to replication in G2. The license is then restored after cell division. By restricting replication licensing to G1, each round of replication must have an intervening cell division, ensuring that DNA is replicated only once per cell cycle.

CDKs inhibit licensing by negatively regulating pre-RC formation

What is the nature of this license? It has now been established that loading of the Mcm2–7 complex represents the license for replication (Blow and Hodgson, 2002; Nishitani and Lygerou, 2002). Therefore, regulation of pre-RC formation is critical for limiting replication to once per cell cycle (Table 1). Since ORC, Cdc6 and Cdt1 are required for Mcm2–7 loading, they are also referred to as licensing factors. The primary regulator of replication licensing is CDK. Licensing is restricted to G1 when CDK levels are low and pre-RCs are allowed to assemble. The increase in CDK activity at the transition to S phase has two important functions for regulating pre-RC formation and function. Firstly, CDK activates factors necessary for origin firing, which concomitantly disassembles the pre-RC, leaving behind an unlicensed origin. Secondly, high CDK activity is inhibitory to the formation of new pre-RCs. Therefore, CDK is responsible for both removing the license and preventing relicensing in S phase. High CDK activity persists until the M–G1 transition, at which time cyclins are rapidly degraded and origins are again competent to be licensed. Consistent with this model, inhibition of

Table 1 Replication licensing – mechanisms for inhibiting pre-RC formation

	S. cerevisiae	S. pombe	Xenopus	*Mammals*
ORC	CDK phosphorylation on chromatin	?	Destabilization of ORC on chromatin	Orc1 dissociation/degradation
Cdc6	Degradation	Degradation	Nuclear Export	Nuclear export CDK phosphorylation on chromatin
Cdt1	Nuclear export	Degradation	Degradation Geminin	Degradation Geminin
Mcm2–7	Nuclear export	?	Reduced affinity for chromatin	Reduced affinity for chromatin CDK inhibition of helicase activity

CDK activity in G2 is sufficient to stimulate rereplication (Broek *et al*., 1991; Dahmann *et al*., 1995; Itzhaki *et al*., 1997). CDK inhibition prior to mitosis is also the mechanism responsible for the physiological rereplication that occurs during certain stages of *Drosophila* development (Follette *et al*., 1998; Su and O'Farrell, 1998; Weiss *et al*., 1998).

CDK phosphorylation exerts its inhibitory effect on pre-RC formation by negatively regulating the action of licensing factors. Each component is controlled independently by CDKs providing redundant pathways that protect against rereplication and genomic instability (Nguyen *et al*., 2001). The most well-studied factor regulated by CDK is Cdc6, which is exported from the nucleus following CDK phosphorylation in vertebrate cells (Saha *et al*., 1998; Petersen *et al*., 1999; Pelizon *et al*., 2000; Delmolino *et al*., 2001) or degraded in yeast (Drury *et al*., 1997, 2000; Jallepalli *et al*., 1997; Elsasser *et al*., 1999). In mammalian cells, a significant fraction of Cdc6 also remains associated with chromatin throughout the cell cycle (Coverley *et al*., 2000; Mendez and Stillman, 2000; Okuno *et al*., 2001; Biermann *et al*., 2002). Phosphorylation of chromatin-bound Cdc6 has no effect on its localization, but inactivates it in some manner (Coverley *et al*., 2002; Alexandrow and Hamlin, 2004). Cdt1 phosphorylation by CDK also targets it for degradation during S phase in mammalian cells (Liu *et al*., 2004; Sugimoto *et al*., 2004), or nuclear export in *S. cerevisiae* (Tanaka and Diffley, 2002). The Mcm2–7 complex travels with the replication fork and hence are displaced from origins upon initiation (Aparicio *et al*., 1997). Mcms that are released from chromatin are exported from the nucleus in *S. cerevisiae* following CDK phosphorylation (Labib *et al*., 1999; Nguyen *et al*., 2000). Mcms remain nuclear throughout the cell cycle in other eukaryotes, but evidence suggest that CDK phosphorylation decreases the affinity of Mcms for chromatin (Coue *et al*., 1996; Hendrickson *et al*., 1996; Fujita *et al*., 1998) and inhibits its helicase activity (Ishimi and Komamura-Kohno, 2001).

Regulation of the ORC complex by CDK has led to the concept of the ORC cycle – the periodic activation and inactivation of ORC during the cell cycle that regulates replication (DePamphilis, 2003). In mammalian cells, Orc1's affinity for chromatin diminishes after origin firing during S phase, at which time it is monoubiquitinated in hamster cells (Li and DePamphilis, 2002) or polyubiquitinated and degraded by the proteasome in mammalian cells (Mendez *et al*., 2002; Tatsumi *et al*., 2003). The S phase CDK, Cyclin A/CDK2, phosphorylates Orc1 (Mendez *et al*., 2002), but it is unclear if this is the mechanism for releasing Orc1 from chromatin and/or targeting it for ubiquitination. During M phase, phosphorylation of Orc1 by Cyclin A/CDK1 prevents Orc1 from rebinding chromatin (Li *et al*., 2004). Although Orc1 binds to DNA if Cyclin A/CDK1 is inhibited, it fails to form a complex with the other ORC subunits on chromatin, suggesting the presence of additional mechanisms that prevent formation of a functional ORC complex in M phase. Orc2–5 levels remain stable and chromatin bound throughout the cell cycle; however, that does not preclude the possibility that CDK phosphorylation regulates the function of other ORC subunits. In yeast, the entire ORC complex remains bound to origins throughout the cell cycle. Rather than disassembling ORC, CDKs exert their inhibitory effect with ORC remaining bound to chromatin. The discovery that ORC phosphorylation is involved in preventing rereplication in *S. cerevisiae* was only apparent when CDK control of Cdc6 and Mcms were ablated as well (Nguyen *et al*., 2001), demonstrating the redundancy provided by the multiple CDK-regulated pathways. In *S. cerevisiae*, CDK is recruited to origins by Orc6 after initiation (Wilmes *et al*., 2004). Preventing CDK recruitment or mutating the CDK phosphorylation sites in Orc2 and Orc6 contributes to rereplication, indicating their role in preventing relicensing (Nguyen *et al*., 2001; Wilmes *et al*., 2004). In a similar fashion, *S. pombe* ORC recruits the M phase CDK, Cyclin B/Cdc2 (Wuarin *et al*., 2002). Disrupting this interaction results in relicensing of origins after S phase and complete genomic reduplication (Wuarin *et al*., 2002). It remains to be determined how phosphorylation of ORC abrogates its function. The role of CDKs in regulating *Xenopus* ORC is less well understood. *Xenopus* ORC can be removed from chromatin by addition of Cyclin A (Hua and Newport, 1998; Findeisen *et al*., 1999). However, *Xenopus* ORC is released after pre-RC assembly independently of kinase activity and origin firing (Rowles *et al*., 1999; Sun *et al*., 2002).

Geminin inhibits replication licensing in higher eukaryotes

Multicellular eukaryotes have evolved a second mechanism to prevent relicensing and rereplication involving the protein Geminin. Geminin forms a dimer that inhibits licensing by directly interacting with Cdt1 (McGarry and Kirschner, 1998; Wohlschlegel *et al*., 2000; Tada *et al*., 2001; Maiorano *et al*., 2004). Although Geminin forms a complex with Cdt1 in

solution (Wohlschlegel *et al*., 2000; Hodgson *et al*., 2002; Yanagi *et al*., 2002), Geminin does not affect Cdt1 loading but rather inhibits recruitment of Mcms by Cdt1 on chromatin (Cook *et al*., 2004). Geminin is loaded onto chromatin as cells enter S phase at about the same time as Cdc45 and persists on chromatin after Cdt1 leaves (Maiorano *et al*., 2004). Recently described structure–function studies suggest that Geminin and Cdt1 execute a bipartite interaction, and confirms biochemical data that Geminin binding prevents Cdt1 from interacting with Mcms by steric hindrance (Lee *et al*., 2004; Saxena *et al*., 2004). Regulation of Geminin in somatic cells resembles CDK activity, being low in G1 and accumulating during S and G2, after which it is ubiquitinated by the anaphase-promoting complex (APC) and degraded during mitosis (McGarry and Kirschner, 1998; Wohlschlegel *et al*., 2000; Nishitani *et al*., 2001). In *Xenopus* and *Drosophila* early embryonic cell cycles, however, Geminin is present throughout the cell cycle (Quinn *et al*., 2001; Hodgson *et al*., 2002; Maiorano *et al*., 2004). In this situation, Geminin is prevented from interacting with Cdt1 during interphase (Tada *et al*., 2001; Cook *et al*., 2004) by being ubiquitinated by the APC (Li and Blow, 2004). However, in contrast to other APC substrates that are polyubiquitinated, Geminin inactivation does not require degradation by the proteasome (Li and Blow, 2004). Since the ubiquitination is transitory, a second modification of Geminin may occur after ubiquitination that inactivates Geminin. Ubiquitination requires CDK-dependent activation of the APC (Li and Blow, 2004). Therefore, in addition to negatively regulating licensing by inhibiting pre-RC formation, CDK positively regulates licensing by inactivating Geminin upon exit from metaphase. Geminin homologues have not been identified in yeast. Since Geminin is also involved in development (Kroll *et al*., 1998; Del Bene *et al*., 2004; Luo *et al*., 2004), it might be unique to multicellular organisms which require additional mechanisms for coordinating DNA replication with cell proliferation during development.

The importance of Geminin in regulating licensing and maintaining genomic stability was demonstrated by knockdown experiments in *Drosophila* and mammalian cells where reduction of Geminin results in rereplication and checkpoint activation that prevents entry into mitosis (Mihaylov *et al*., 2002; Melixetian *et al*., 2004; Zhu *et al*., 2004). Inhibition of relicensing by Geminin is specific to S phase and/or G2 (Ballabeni *et al*., 2004). In M phase, inhibiting CDK activity is sufficient to cause relicensing regardless of Geminin status (Ballabeni *et al*., 2004). Therefore, Geminin's inhibitory function may be specific for S phase when CDK levels are not high enough to suppress relicensing, whereas in mitosis CDK is primarily responsible. It has been suggested that, in addition to its role as a negative regulator of licensing in S phase, Geminin plays a second role as a positive regulator of licensing during M phase by binding to and stabilizing Cdt1 (Ballabeni *et al*., 2004). While Geminin knockdown in S phase induces rereplication, knockdown of Geminin in M phase resulted in impaired pre-RC formation during the ensuing cell cycle. It will be interesting to determine if post-translational modification of Geminin is responsible for the different activities of Geminin. It is important to mention that depletion of Geminin in cycling *Xenopus* extracts fails to stimulate rereplication (McGarry and Kirschner, 1998). However, Geminin depletion does result in a G2 arrest during embryonic cell cycles dependent on Chk1 (McGarry, 2002) similar to that seen in *Drosophila* and mammals. Therefore, it is possible that a very low level of reinitiation occurs that is sufficient for checkpoint activation, but not significant enough to be detected. Another possibility is the presence of Geminin-independent mechanisms that prevent rereplication, which are unique to *Xenopus* embryonic cell cycles.

Checkpoints ensure a smooth progression through S phase

Defects in replication initiation lead to halting of cell cycle progression through what are known as checkpoint pathways (Nyberg *et al*., 2002; Cobb *et al*., 2004). Checkpoints ensure that cell cycle events have occurred correctly and completely before proceeding to the next stage of the cell cycle. DNA damage during G1, for example, inhibits entry into S phase and thereby prevents replication of damaged DNA. Furthermore, checkpoints facilitate repair and induce programmed cell death in the face of irreparable damage. As a result, cells defective for checkpoint proteins are highly sensitive to damaging agents and cellular stress. A subset of checkpoint proteins are essential for viability in the absence of exogenous damage, indicating that checkpoint proteins are also required to deal with endogenous stresses that occur during normal cell proliferation. In this section, we will cover checkpoints that are activated by disruption of the replication process; in particular, checkpoints activated by stalled forks and inappropriate licensing. Checkpoints activated by irradiation and double-stranded breaks will not be discussed; however, the different checkpoints often activate parallel and overlapping pathways that feed into common downstream effectors. We also defer discussion of specialized DNA polymerases involved in tranlesion synthesis to other reviews (Friedberg *et al*., 2002; Hubscher *et al*., 2002).

Stalled replication forks

Elongating replication forks stall when nucleotide pools are depleted or when they encounter lesions in DNA such as those caused by the alkylating agent MMS. Replication forks appear to be the sensor for detecting damage caused by alkylating agents as checkpoints are activated only during S phase regardless of when MMS is added during the cell cycle (Tercero and Diffley, 2001; Tercero *et al*., 2003). Experiments in *Xenopus* also show that replication is required for checkpoint activation during S phase (Lupardus *et al*., 2002; Stokes *et al*., 2002). Therefore, damage may go undetected until a replication fork collides with the lesion. Stalled replica-

tion forks signal a cascade of events that inhibit origin firing, stabilize stalled forks and prevent entry into mitosis (Nyberg *et al*., 2002). The most important function for maintaining cell viability is the stabilization of stalled replication forks (Desany *et al*., 1998; Tercero *et al*., 2003). Maintaining the integrity of forks is imperative for survival because restarting stalled forks is the primary mechanism for recovering from damage (Lopes *et al*., 2001; Tercero and Diffley, 2001). Therefore, stalled replication forks are both the primary sensor and target of checkpoints. In checkpoint-deficient yeast strains, stalled replication forks undergo irreversible transformations to abnormal structures that are unable to restart DNA synthesis (Lopes *et al*., 2001). Some of these structures resemble substrates for recombination intermediates, suggesting that in the absence of fork stabilizing mechanisms inappropriate recombination occurs, leading to chromosome instability (Sogo *et al*., 2002). Similar findings are observed in mammalian cells where checkpoint inhibition releases replication factors from chromatin, presumably due to fork collapse, and prevents stalled forks from resuming synthesis (Dimitrova and Gilbert, 2000).

Recent work, mainly carried out in yeast, has begun to clarify the mechanism by which stalled forks activate these pathways (Figure 3). The molecular aberration that is initially detected is single-stranded DNA coated by RPA. Stalled forks generate stretches of single-stranded DNA (Sogo *et al*., 2002) that result in accumulation of RPA on chromatin (Michael *et al*., 2000; Lupardus *et al*., 2002; Zou and Elledge, 2003). The checkpoint kinase ATR in humans or Mec1 in *S. cerevisiae* is then recruited to RPA-ssDNA by ATR interacting protein (ATRIP) or its homologue in yeast Ddc2 (Zou and Elledge, 2003). ATR is a central player in multiple checkpoint pathways that are activated by damage in addition to those caused by stalled forks, suggesting that different forms of insults are processed to a common intermediate such as single-stranded DNA. Once recruited, ATR phosphorylates and activates the 'effector' kinase Rad53 in yeast or Chk1 in mammals and *Xenopus*, whose activity is required for mediating downstream events including inhibition of late origin firing, stabilization of replication forks and prevention of entry into M phase (Nyberg *et al*., 2002). Rad53 and Chk1 are recruited to stalled forks by newly characterized 'adaptor' proteins. Interestingly, two of these proteins, Mrc1 and Tof1, travel with the normal elongating replication fork and are loaded after Cdc45 during origin firing (Katou *et al*., 2003; Osborn and Elledge, 2003). The metazoan homologue of Mrc1, Claspin, is similarly loaded at origins during replication initiation (Lee J *et al*., 2003) and human Claspin has been shown to form a ring structure in solution (Sar *et al*., 2004), suggesting that it may encircle DNA and travel with the replication fork. No homologue of Tof1 in higher eukaryotes has been described thus far. Mrc1 and Claspin are substrates for Mec1 and ATR, respectively, and their phosphorylation is required for Rad53 (Alcasabas *et al*., 2001; Osborn and Elledge, 2003; Zhao *et al*., 2003) and Chk1 recruitment and activation (Kumagai and Dunphy, 2003; Chini and Chen, 2004; Lin *et al*., 2004). Other components of the replication fork also have roles in mediating the checkpoint signal in addition to their function in initiation and elongation such as Dpb11 (Araki *et al*., 1995), Drc1/Sld2 (Wang and Elledge, 1999), DNA pol α (Michael *et al*., 2000) and DNA pol ε (Navas *et al*., 1995). It is unclear how these checkpoint pathways stabilize stalled replication forks. Checkpoints may prevent the premature disassembly of the replication machinery as loss of Mec1 results in dissociation of Cdc45 (Katou *et al*., 2003) and the replicative polymerases (Cobb *et al*., 2003). It has also been postulated that Rad53 may prevent decoupling of the replicative polymerases that occur when forks stall (Sogo *et al*., 2002).

In addition to propagating the checkpoint signal, Mrc1 and Tof1 play a role in pausing replication forks in the presence of hydroxyurea (Katou *et al*., 2003). Failure to pause in Mrc1 and Tof1 mutants results in the migration of the replication machinery along the chromosome without DNA synthesis. However, it is not known whether this also occurs in response to alkylating agents which present a physical barrier to fork migration. The finding that the replication fork has evolved to incorporate checkpoint proteins suggests that fork pausing and restarting may occur during a normal S phase as the replication machinery encounters higher-order chromatin structures, or in response to endogenous sources of damage such as free radicals generated during normal metabolic processes. In support of this

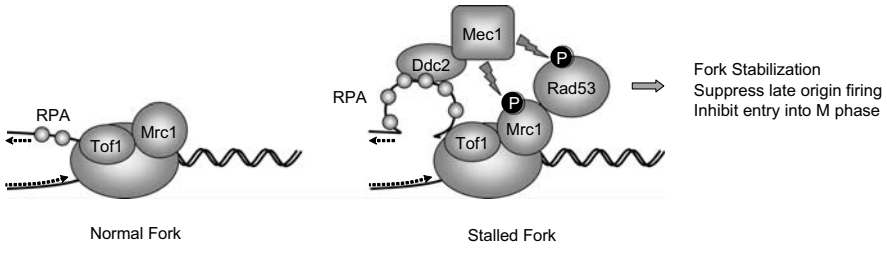

Figure 3 Checkpoint activated in response to stalled replication forks. Nucleotide depletion or DNA damage causes replication forks to stall, resulting in accumulation of single stranded DNA. RPA-coated single-stranded DNA recruits Mec1 through Ddc2 to phosphorylate downstream components of the checkpoint pathway that function to stabilize replication forks and halt cell cycle progression. Tof1 and Mrc1 are components of the normal replication fork that are necessary for propagating the checkpoint signal. Depicted in the figure are the names of proteins from *S. cerevisiae*; however, most of the proteins are conserved in all eukaryotes

hypothesis, ATR and Chk1 are essential genes (Brown and Baltimore, 2000; Liu Q *et al*., 2000b), and conditional deletion of ATR during S phase results in chromosomal breakage at fragile sites in the mammalian genome (Casper *et al*., 2002). In *Xenopus*, ATR is recruited to chromatin in an unperturbed cell cycle (Hekmat-Nejad *et al*., 2000) and its activity regulates origin firing by modulating Cdc7 and CDK activity (Shechter *et al*., 2004). *S. cerevisiae* Mec1 is also required for preventing chromosomal breakage in slow-replication progression zones in the absence of damaging agents (Cha and Kleckner, 2002).

Inappropriate replication licensing and initiation

Rereplication can be produced in cancer cells by overexpression of Cdt1 and Cdc6 (Vaziri *et al*., 2003). The rereplicated regions are predominantly enriched in segements of the chromosomes that replicate early in S phase and are accompanied by ATM/ATR- and Chk2-dependent activation of p53, characteristic of DNA damage. The resulting inhibition of the cell cycle and induction of apoptosis make it difficult for p53+ cells to survive with enough rereplicated DNA, so that the phenomenon is selectively observed in p53− cancer cells.

Rereplication caused by depletion of Geminin in mammalian cells also activates a checkpoint that prevents cell cycle progression. Analysis of downstream checkpoint proteins indicate that rereplication activates the G2/M checkpoint mediated by the inhibition of CDC25C and CDK by Chk1 and the ATM/ATR kinases (Melixetian *et al*., 2004; Zhu *et al*., 2004). The appearance of gamma H2AX and Rad51 foci is characteristic of DNA damage and repair (Shiloh, 2003), suggesting that rereplication generates intermediates resembling damage such as double-stranded breaks and/or stalled forks. However, the exact nature of the molecular signal that activates the checkpoint remains to be determined, nor is it clear as to why p53 or Cdc25C are differentially used as effectors in response to rereplication induced by Cdt1 overexpression *versus* geminin depletion. The purpose of these pathways is to maintain genomic integrity as ablation of these checkpoints results in a catastrophic mitosis with chromosomal breakage (Melixetian *et al*., 2004; Zhu *et al*., 2004). Therefore, loss of Geminin may predispose cells to chromosomal instability and cancer. In addition to relicensing, the effects of insufficient licensing were examined in various mammalian cell lines by overexpressing a nondegradable form of Geminin (Shreeram and Blow, 2003). Interestingly, cancer cell lines and primary cell lines responded differently to the inhibition of licensing. Cancer cell lines entered S phase and underwent apoptosis with activation of S phase checkpoints. However, a primary cell line arrested in G1 with low levels of CDK activity. Therefore, the primary cell line has a 'licensing checkpoint' that monitors licensing prior to S phase, which is missing in the cancer cell lines examined. It will be interesting to determine the signals that trigger checkpoint activation in response to inappropriate or inadequate licensing, and whether it contributes to development of cancer.

Replication proteins link S phase to the rest of the cell cycle

A growing body of evidence has associated replication factors with other cellular processes, probably as a means of communicating replication status to the cell cycle. A good example is the replication proteins that are required for both initiation and checkpoint activation leading to cell cycle arrest. Although ORC is required for localizing pre-RC components to origins in all eukaryotes, recent studies have identified additional functions for several of the ORC subunits. Orc6 has been shown to be involved in chromosome segregation and cytokinesis (Prasanth *et al*., 2002; Chesnokov *et al*., 2003) and Orc2 implicated in chromosome condensation and centrosome duplication (Prasanth *et al*., 2004). These functions occur outside the context of the six-subunit ORC complex, suggesting that the different subunits may have modular functions which can be incorporated into other complexes. Consistent with this, subsets of the six human ORC subunits have been reported to be present in nonproliferating tissues like the heart or the brain (Thome *et al*., 2000). ORC is also involved in heterochromatin formation and transcriptional silencing separable from its role in replication (Foss *et al*., 1993; Fox *et al*., 1995). Geminin has been shown to be important for regulating the function of replication proteins and transcription factors during development (Del Bene *et al*., 2004; Luo *et al*., 2004), suggesting an important, and relatively unexplored, network of interactions that couple replication to transcriptional programs. No doubt, more of these relationships will be uncovered in the future.

Concluding remarks

Since the discovery of the structure of DNA 50 years ago which produced a mechanism for copying the genome, and the purification of DNA polymerase which provided the molecular means to accomplish the task, scientists have been trying to understand how the two come together to faithfully duplicate the genome with every cell division. We now know that a highly regulated pathway of protein interactions need to occur before polymerase is positioned to begin DNA synthesis. Although we continue to identify many of the players involved, we know very little about the biochemical activities of these proteins at an origin, and how they contribute at the molecular level to the replication process. A major challenge in the future will be to decipher these mechanisms using a combination of structural studies and biochemistry. Another fundamental question that remains unanswered is what are the genetic and epigenetic elements that define an origin? Answering this question will be aided by the completed

sequence of several eukaryotic genomes, as we are now capable of performing high resolution mapping across chromosomes to identify and analyse origins on a larger scale. These kinds of studies may also uncover clues to understanding the problem of origin timing and spacing, and provide insight into how origin selection and replication fork progression are influenced by factors like gene density, epigenetic changes and the genetic background of the cell. The recent breakthrough of RNAi technology has provided scientists with the ability to perform genetics in mammalian cells. Genetic studies in yeast have been instrumental for understanding the replication process, and, although many of the proteins involved are conserved, yeast and humans are separated by a billion years in evolutionary distance. Genetic screens will be a powerful resource for characterizing processes unique to mammalian replication.

The replication initiation factors and regulators described here are expected to become important for developing new therapies for cancers. The interaction of the disorders of replication initiation with checkpoint pathways clearly needs further exploration. Given that mutations in checkpoint pathways and overexpression of replication initiator proteins are seen in human malignancies, it is likely that an understanding of these interactions will reveal how they contribute to genetic instability in cancer cells. Conversely, efforts are under way to take advantage of the checkpoint pathway anomalies in cancers for therapeutic benefit. Levels and activities of replication factors and their regulators might then have prognostic significance for such therapeutic interventions. Finally, some of the replication initiator proteins have been implicated in the replication of viral episomes in cancer cells (Dhar *et al*., 2001; Schepers *et al*., 2001). Further work is needed to understand this dependence and to exploit it to develop therapies for cancers that are caused by episomally maintained viruses.

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